EXHIBIT A

Hapten-Induced Model of Chronic Inflammation and Ulceration in the Rat Colon

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We have developed a simple and reproducible rat model of chronic colonic inflammation by the intraluminal instillation of a solution containing a "barrier breaker" and a hapten. Administration of the hapten 2,4,6-trinitrobenzenesulfonic acid (5–30 mg) in 0.25 ml of 50% ethanol as the "barrier breaker" produced dose-dependent colonic ulceration and inflammation. At a dose of 30 mg, trinitrobenzenesulfonic acid/ethanol-induced ulceration and marked thickening of the bowel wall persisted for at least 8 wk. Histologically, the inflammatory response included mucosal and submucosal infiltration by polymorphonuclear leukocytes, macrophages, lymphocytes, connective tissue mast cells, and fibroblasts. Granulomas were observed in 57% of the rats killed 3 wk after induction of inflammation. Langhan's-type giant cells were also observed. Segmental ulceration and inflammation were common. The characteristics and relatively long duration of inflammation and ulceration induced in this model afford an opportunity to study the pathophysiology of colonic inflammatory disease in a specifically controlled fashion, and to evaluate new treatments potentially applicable to inflammatory bowel disease in humans.

Research on the etiopathogenesis of inflammatory bowel disease and testing of potential therapeutic agents have been hampered by the paucity of reproducible and histopathologically relevant animal models of chronic inflammation. An animal model of spontaneous colitis has recently been described (cotton-top tamarins) in which the inflammation is chronic and responds to sulfasalazine treatment (1). Unfortunately, this endangered species is not widely available, thus limiting its usefulness. Several animal models of acute intestinal inflammation resembling ulcerative colitis have been developed and are used extensively (2–7). Chronic intestinal inflammation and ulceration has also been produced in laboratory animals using a variety of techniques. Mee et al. (8) immunized rabbits with the enterobacterial antigen of Kunin and then injected soluble immune complexes intravenously and irritated the colon with dilute formalin. The result was a chronic inflammatory response with ulcerations in the region in which the formalin was applied (distal colon). Sartor et al. (9) recently described a rat model of chronic, granulomatous inflammation induced by intramural injection of bacterial cell wall fragments. Although this model offers several advantages over others, it requires a surgical procedure and the resulting "disease" was not characterized by mucosal ulceration or features such as diarrhea and weight loss. Nemirovsky and Hugon (10) described a model of autoimmune enterocolitis in guinea pigs induced by alloimmunization with a mucosal protein. This model was characterized by chronic, granulomatous inflammation and ulceration of the distal ileum and colon. Stewart et al. (11) induced chronic ulcerative colitis in dogs by daily administration of indomethacin or cinchophen. Although each of these models has some features that are characteristic of human inflammatory bowel disease, they all have significant disadvantages, such as requirements for surgery, limited duration of the inflammatory response, or lack of reproducibility and, in some instances, the use of a relatively expensive species of laboratory animal.

In the present study, we sought to develop a simple and reproducible model of chronic intestinal inflammation. The approach used was based on the hypotheses of Ward (12) and Shorter et al. (13), which presume that in Crohn's disease, an increase in mucosal permeability results in entry to the lamina propria of a luminal antigen that is not adequately cleared by the mucosal immune system. Thus, we instilled into the distal colon of the rat a solution containing ethanol (the "barrier breaker") and trinitrobenzenesulfonic acid (TNB). Trinitrobenzenesulfonic acid is a hapten, but when coupled to a substance of high molecular weight (such as tissue proteins), it has been shown to elicit immunologic responses (14,15). Ethanol has been shown to cause widespread acute mucosal damage when instilled into the distal colon of the rat at a concentration of 30% (16). The combined administration of TNB and ethanol resulted in the development of severe, transmural, granulomatous inflammation of the distal colon. Ulceration of the colonic mucosa persisted for at least 8 wk. This model shares many of the histopathological and clinical features of human Crohn's disease and may be useful for the study of the etiopathogenesis of chronic intestinal inflammation as well as providing an inexpensive model suitable for assessing potential treatments.

Materials and Methods

Animals

Virgin female Sprague-Dawley rats (250-300 g), obtained from Canadian Breeding Farms (Montreal, Canada), were used in this study. They were maintained in a restricted access room with controlled temperature (23°C) and light/dark (14 h:10 h) cycle. The animals were housed in rack-mounted, wire cages with a maximum of 6 animals per cage. Standard laboratory pelleted formula and tap water were provided ad libitum.

Induction of Chronic Inflammation

The rats were randomized into treatment groups, then lightly anesthetized with ether. A rubber catheter (OD, 2 mm) was inserted rectally into the colon such that the tip was 8 cm proximal to the anus, approximately at the splenic flexure. 2,4,6-Trinitrobenzenesulfonic acid (Sigma Chemical Co., St. Louis, Mo.) dissolved in 50% ethanol (vol/vol) was instilled into the lumen of the colon through the rubber catheter (total volume, 0.25 ml). The instillation procedure required ~5 s to complete. Initially, a dose-response study was performed in which TNB was administered at doses of 5, 10, 20, or 30 mg per rat in the 50% ethanol vehicle (n = 5 per group). The rats were killed 2 wk later for assessment of damage. Based on the results of this dose-response study, the dose of TNB used in subsequent experiments was 30 mg per rat. In control

experiments, rats received (administered as before) 0.25 ml of either 50% ethanol alone, 30 mg of TNB in 0.9% saline, or 0.9% saline alone. Control rats were always housed in separate cages from TNB-treated rats.

Assessment of Colonic Inflammation and Damage

At various times (24 h and 1-8 wk) after intracolonic administration of 30 mg of TNB in 50% ethanol or one of the control solutions, 5 or more rats from each treatment group were randomly selected and killed by an injection of sodium pentobarbital (90 mg/kg i.p.). The distal colon was removed, opened by a longitudinal incision, pinned out on a wax block, and assigned a code number. The colon was immediately examined under a stereomicroscope and any visible damage was scored on a 0-5 scale (Table 1) by two independent observers blinded to the treatment. There was a highly significant linear correlation between the scores assigned by the two observers (r = 0.98, p < 0.001). After scoring, three tissue samples (2 × 10 mm) were excised from each colon. When no grossly visible inflammation was present, the samples were taken from the regions ~1, 3, and 6 cm proximal to the anus. When visible ulceration or inflammation was present, at least one of the samples was taken from the affected region. In some studies, samples for histologic assessment were also taken from the transverse colon, ileum, jejunum, duodenum, liver, kidneys, and spleen. The tissue samples were fixed in Parfix (Fisher Scientific, Pittsburgh, Pa.) and processed routinely before embedding in paraffin. Sections (7 μm) were stained with hematoxylin and eosin or acridine orange/hematoxylin. The acridine orange/hematoxylin staining procedure allowed for identification and separation of mucosal and connective tissue mast cells. Histologic assessment by light microscopy was performed in a blinded fashion on coded slides. Thickness of the colon wall was determined by measuring the distance from the serosal surface to the luminal surface of the mucosa. Measurements were made at 1-mm intervals along the entire length of each section (at least two sections per rat) and were restricted to regions where glands were continuous in sections throughout the depth of the mucosa. The

Table 1. Criteria for Scoring of Gross Morphologic Damage

Score	Gross morphology
0	No damage
1	Localized hyperemia, but no ulcers.
2	Linear ulcers with no significant inflammation.
3	Linear ulcer with inflammation at one site.
4	Two or more sites of ulceration and/or inflammation.
5	Two or more major sites of inflammation and ulceration or one major site of inflammation and ulceration extending >1 cm along the length of the colon.

"Inflammation" was defined as regions of hyperemia and bowel wall thickening. Colons were examined under a stereomicroscope independently by 2 observers who were unaware of the treatment.

presence of epithelioid granulomas in these sections was also noted. Epithelioid granulomas were defined, using the criteria of Surawicz and Belic (17), as discrete collections of at least five epithelioid cells, with or without accompanying giant cells, and without caseation necrosis or foreign bodies. All granulomas were examined with polarizing microscopy to detect the presence of foreign bodies.

Colonic myeloperoxidase (MPO) activity and weight of the distal 8 cm of the colon were determined as further indices of inflammation. Myeloperoxidase is an enzyme found predominantly in the azurophilic granules of polymorphonuclear leukocytes (neutrophils) and has been used as a quantitative index of inflammation in several tissues, including the intestine (5,18–20). Subsequent to weighing on an analytical balance, the distal 8-cm segment of the colon was suspended in 0.5% hexadecyltrimethylammonium bromide (pH 6.0; 50 mg of tissue per milliliter) and was then homogenized for 15 s using a Polytron generator (Brinkman Instruments, Rexdale, Ontario, Canada). After freeze-thawing the homogenate three times, the tissue levels of MPO activity were determined using the technique described by Bradley et al. (18), utilizing 0.0005% hydrogen peroxide as a substrate for the MPO. A unit of MPO activity was defined as that converting 1 μmol of hydrogen peroxide to water in 1 min at 22°C. The MPO assays were performed in a blinded fashion on coded tubes.

Statistical Methods

Unless otherwise stated, data are expressed as mean ± SEM. Parametric data were analyzed using the Student's two-tailed t-test for unpaired observations. Linear regression analyses were performed using the Statgraphics software package, version 2.0. Nonparametric data were analyzed with the Mann–Whitney U-test. With all statistical analyses, an associated probability (p value) of ≤5% was considered significant.

Results

Dose-Response Study

The severity of colonic damage induced by TNB increased with the amount administered (Figure 1). Rats that received the lowest dose of TNB (5 mg) had damage scores, colon weights, and tissue levels of MPO activity that were not significantly different from control animals treated only with the 50% ethanol vehicle. With doses of TNB of 10–30 mg, tissue levels of MPO activity, colon weights, and damage scores increased in a dose-related manner. There was a highly significant (p < 0.01) linear correlation between the dose of TNB administered and the damage scores (r = 0.85), colon weight (r = 0.76), and tissue levels of MPO activity (r = 0.73).

Time-Course Study

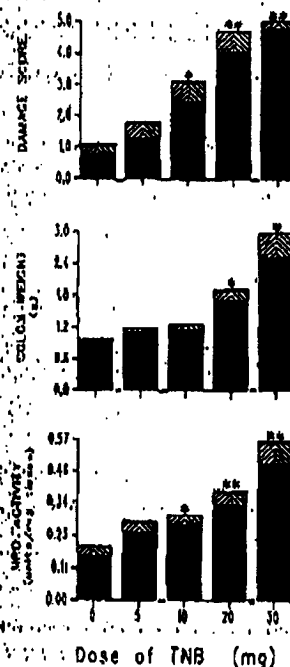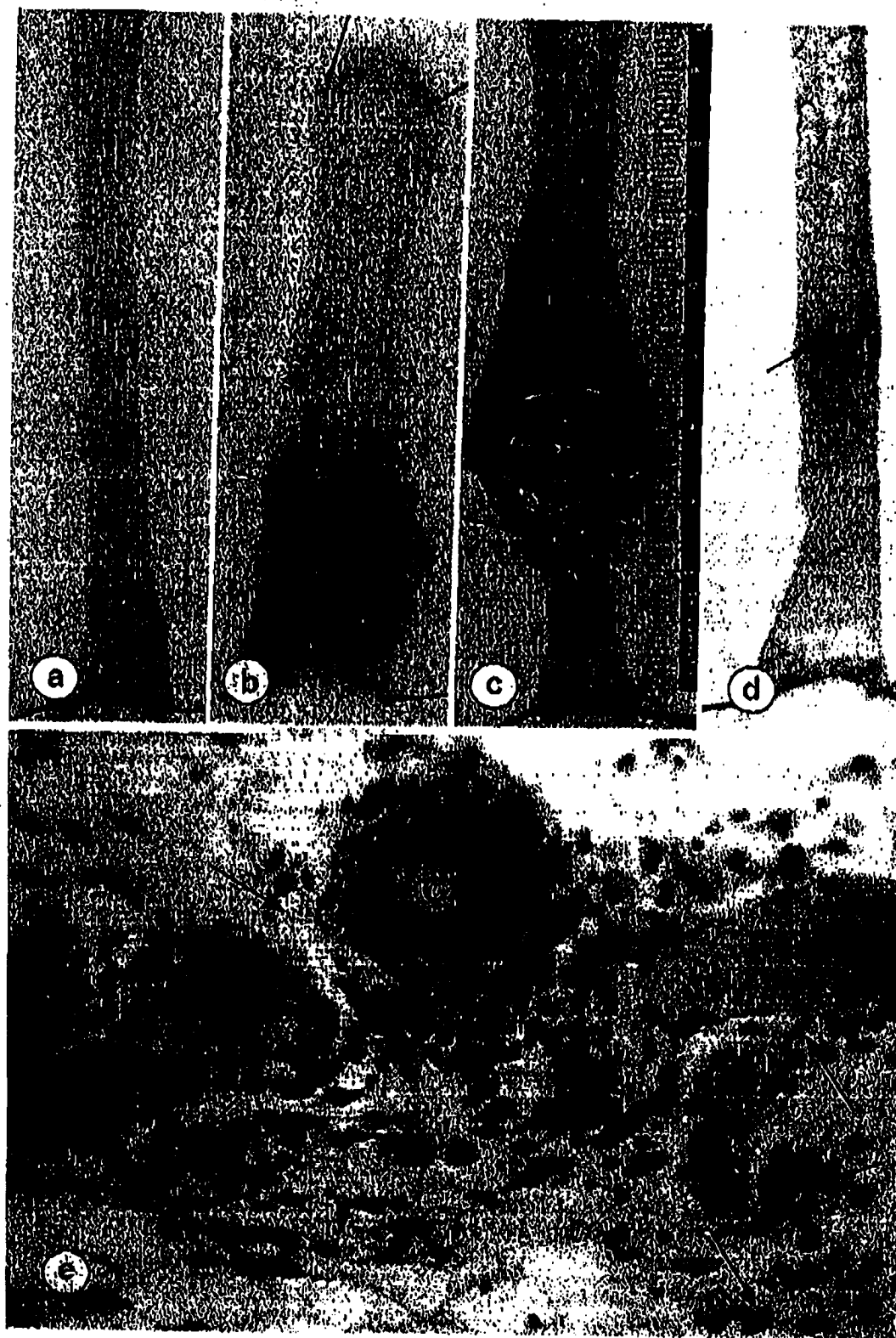Intraluminal administration of 0.9% saline alone did not produce any macroscopically or histo-

Figure 1. Data are presented as the mean ± SEM. Rats were killed 2 wk after intracolonic administration of 50% ethanol containing 0–30 mg of TNB. Colonic damage was scored on a 0 (normal) to 5 (severe) scale by 2 independent observers. Tissue MPO activity is expressed as units of activity per milligram of tissue. Colon weight represents the wet weight of the distal 8 cm, in grams. n = 5 per group. Asterisks denote groups that differ significantly from the control (0 mg TNB) group (* p < 0.05; ** p < 0.01; Student's t-test for MPO and colon weight; Mann–Whitney U-test for damage scores).

logically detectable damage. In the control groups treated with 50% ethanol or 30 mg of TNB in saline, acute colonic damage characterized by hemorrhage and bowel wall thickening was present 1 day after administration. However, colon weight, damage scores, and tissue levels of MPO activity were similar to the levels observed in saline-treated rats by 1 wk after administration of either solution.

All of the animals that received 30 mg of TNB in 50% ethanol developed areas of grossly visible bowel wall thickening, inflammation, and ulcers. Such ulcers were observed up to 8 wk after administration of TNB/ethanol. The sites of inflammation and ulceration varied from the perirectal region to 7 cm proximal to the anus. There were often two or more separate sites of inflammation in the distal colon. No damage was detected proximal to the splenic flexure. In addition to severe, transmural inflammation, segmental pericolic accumulations of mesenteric fat and fibrinous adhesions to the small bowel and uterine horns were frequently observed. The ulcers appeared as white foci or lines and were
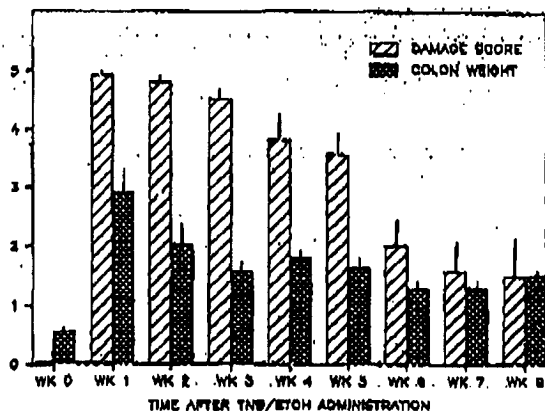
Figure 3. The effects of intracolonic administration of 30 mg of TNB in 0.25 ml of 50% ethanol on colon weight (distal 8 cm) and damage score 1-8 wk after administration. Each bar represents the mean ± SEM of at least 5 animals (for exact n values see Table 3). The "Wk 0" group refers to the overall mean ± SEM for rats that received saline in place of TNB and were killed 1-8 wk later. All groups of data for the TNB-treated rats were significantly (p < 0.01) greater than the corresponding control (saline-treated) group.

Table 2. Effects of Trinitrobenzenesulfonic Acid/Ethanol on Colonic Myeloperoxidase Activity: Time-Course Study

Time after TNB/ethanol	Number in group	MPO (U/mg)
0 (Control)	9	0.81 ± 0.02
1 wk	26	1.02 ± 0.10[n]
2 wk	14	0.98 ± 0.14[a]
3 wk	9	1.11 ± 0.10[a]
4 wk	6	1.28 ± 0.21[a]
5 wk	9	0.75 ± 0.07[a]
6 wk	6	0.89 ± 0.06[n]
7 wk	8	0.72 ± 0.16[a]
8 wk	6	0.57 ± 0.66

MPO, myeloperoxidase; TNB, trinitrobenzenesulfonic acid. Data are expressed as the mean ± SEM. All animals received 30 mg of trinitrobenzenesulfonic acid in 50% ethanol intracolonically, except for the control group, which received 0.9% saline. Myeloperoxidase activity of the distal 8 cm of colon is expressed as the units of activity per milligram of tissue. ^{a} p < 0.01 by Student's t-test when compared with control group.

rounded by thickened, inflamed mucosa. Frequently, a localized "cobblestone" appearance resulted when regions of mucosa were completely encircled by ulcers (Figure 2b). Linear ulcers were 1 to 3 mm wide and often exceeded 1 cm in length (Figure 2d). Extensive ulcers often involved the entire circumference and extended more than 3 cm along the length of the colon (Figures 2b and 2c). No abnormalities were observed in histologic specimens of liver, spleen, kidney, ileum, jejunum, and duodenum. Diarrhea was observed in >90% of the rats killed 1-3 wk after TNB/ethanol administration, but was never seen in the control groups. Incomplete bowel obstruction (marked narrowing of the lumen of the colon adjacent to inflamed sites) with proximal dilatation of the bowel, was frequently observed at all times studied (>50% of rats killed 1-8 wk after TNB/ethanol), but perforation did not occur. Mortality in rats treated with 30 mg of TNB/ethanol was 7%, with the majority of deaths occurring during the

first 2 wk after administration of the hapten. The rats that died displayed grossly distended colons, with no perforation. An 8%-10% decrease in total body weight was observed during the first week after TNB/ethanol, after which growth rates returned to normal. (3-5 g/day). Significant weight loss was not observed in any of the three control groups.

After TNB/ethanol administration, all three of the employed indices of damage and inflammation were significantly (p < 0.01) elevated above the levels observed in animals that received any of the three control treatments. Damage scores and colon weight remained significantly (p < 0.01) elevated above control levels for 8 wk after administration of TNB/ethanol (Figure 3), and tissue levels of MPO activity were significantly (p < 0.01) higher than control levels during weeks 1-7 (Table 2).

The histologic appearance of tissues examined 1 day after administration of TNB/ethanol did not differ from that of tissues taken 1 day after administration of 50% ethanol alone or of 30 mg of TNB in saline, although the extent of ulceration was greatest in the group that received TNB/ethanol. There was

Figure 2. Panels a-d are photographs of colons from rats given 30 mg of TNB in 0.25 ml of 50% ethanol or the vehicle alone (intracolonically) and killed at various times afterwards. a. Rat killed 2 wk after administration of the 50% ethanol vehicle. This colon was given a damage score of 0. b. Rat killed 2 wk after administration of TNB/ethanol. Note the extensive ulceration involving the distal 4 cm of the colon. Arrow indicates an ulcer surrounded by grossly normal tissue. This colon was given a damage score of 8. c. Rat killed 4 wk after administration of TNB/ethanol. Note the grossly visible enlargement of the colon and the large ulcer. This colon was given a damage score of 6. d. Rat killed 6 wk after administration of TNB/ethanol. Arrow indicates a discrete, transverse ulcer. This colon was given a damage score of 3. e. Light micrograph of a small serosal granuloma in a sample of colon taken from a rat 4 wk after intracolonic administration of TNB/ethanol. This granuloma has a central Langhan's-type giant cell (G) associated with hemosiderin-containing macrophages (small arrows) and connective tissue mast cells (large arrows). Magnification, ×720 (acridine orange/hematoxylin).
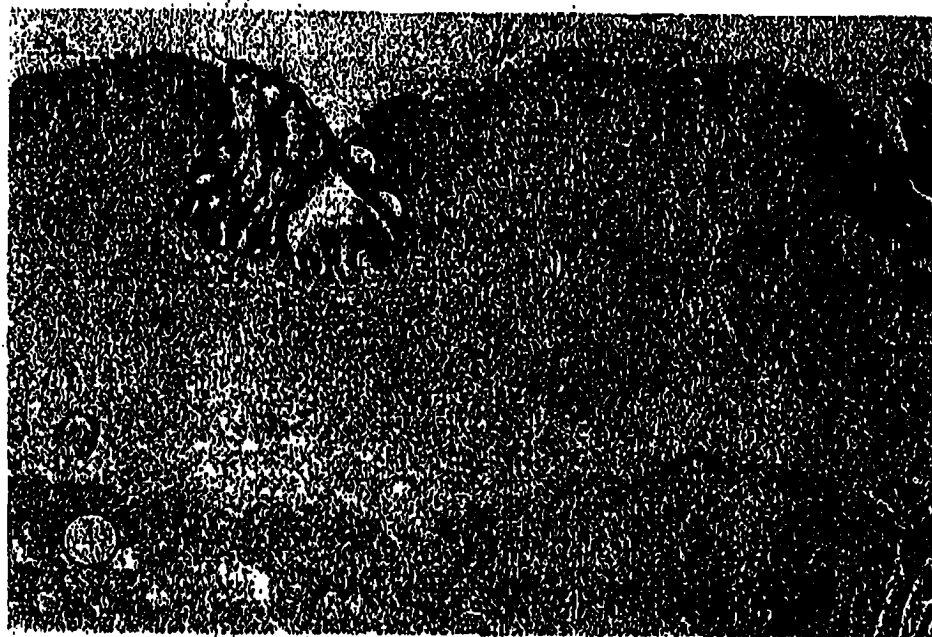
Figure 1. Ulceration and inflammation of the colon 2 wk after intracolonic administration of TNB in 50% ethanol. Areas of ulceration are separated by intact glandular mucosa. Magnification, ×48 (hematoxylin & eosin).

extensive mucosal damage characterized by edema, hemorrhage, epithelial exfoliation, and infiltration of polymorphonuclear leukocytes. The muscularis mucosae remained intact.

At both 1 and 2 wk after administration of TNB/ethanol, the inflammation extended through the mucosa and submucosa, and often included the muscularis externa (Figure 4). Extensive infiltration by polymorphonuclear leukocytes, macrophages, eosinophils, fibroblasts, connective tissue mast cells, and lymphocytes was apparent. The mucosa adjacent to ulcers showed extensive crypt distortion (Figure 5). Granulomas, usually in the submucosa and serosa, were most frequently observed in the rats killed 1–3 wk after TNB/ethanol (Table 3). Foreign-body giant cells were occasionally present in the superficial (most luminal) regions of the inflammatory cell mass. Tissues examined 3–5 wk after administration of TNB/ethanol showed progressively increased inflammatory activity in the muscularis externa and involvement of the serosa. In some regions, the muscularis was obliterated by the inflammatory cell mass.

By 3 wk after TNB administration, polymorphonuclear leukocytes were primarily located within the superficial regions of the ulcers. Connective tissue mast cells were most prevalent during weeks 3 and 4 (the periods of most extensive chronic inflammation) and were primarily located in the serosa and submucosa surrounding the inflammatory cell mass. Connective tissue mast cells and hematin-containing

macrophages were often located at the periphery of granulomas (Figure 2a).

As outlined in Table 3, the mean thickness of the bowel wall at sites of grossly visible ulceration/inflammation was increased by up to 225% (compared with normal regions of the same colon) in rats killed 1–8 wk after administration of TNB/ethanol. In some sections of ulcerated tissue the bowel wall was increased to more than eight times the thickness observed in sections of grossly normal tissue.

Discussion

These results demonstrate that intracolonic administration of the hapten TNB mixed with a "barrier breaker" (50% ethanol) results in long-lasting ulceration and inflammation of the rat colon. This damage was characterized by marked thickening of the colonic wall, infiltration of polymorphonuclear leukocytes (assessed histologically and by MPO activity), and granuloma formation. Such chronic inflammation was not produced by administration of the hapten or the barrier breaker alone. These results are consistent with the hypotheses of Ward (12) and Shorter et al. (13) that chronic inflammation of the intestine may occur as a consequence of increased permeability of the mucosa to a luminal antigen which cannot be readily cleared by the immune system. Using a monoclonal antibody to TNB for immunohistochemical localization studies,
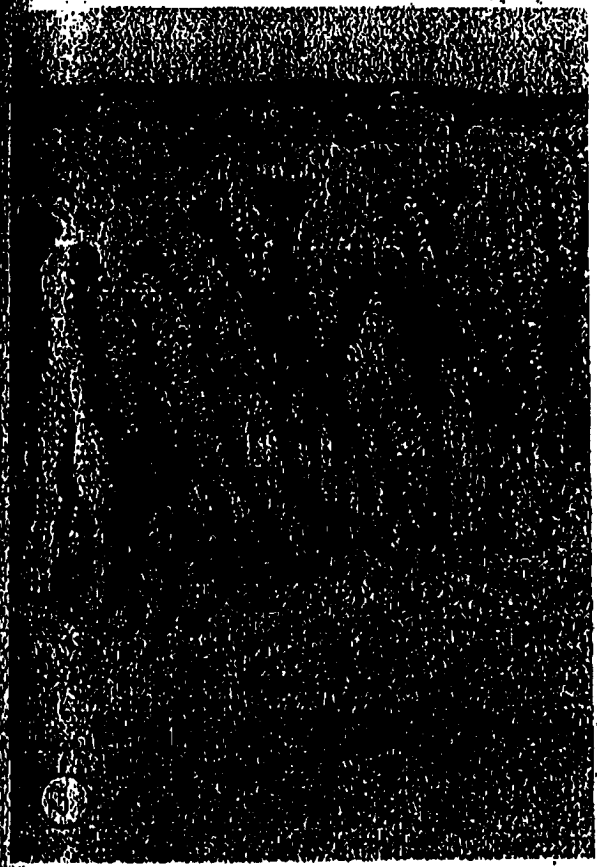
Figure 5: Colonic mucosa over a site of chronic inflammation from rat killed 3 wk after intracolonic administration of 30 mg of TNB in 50% ethanol. Note the mucosal thickening, distorted crypt architecture, and preservation of goblet cells. The mucosa is covered primarily by mucus and polymorphonuclear leukocytes. Magnification, ×85 (hematoxylin and eosin).

We have observed TNB in colonic tissue for up to 5 wk after induction of colitis (unpublished).

Several features of this model make it an attractive one for study of both the pathophysiology and treatment of intestinal inflammation. First, the inflammation is induced by a single intraluminal administration, with no requirement for previous sensitization of the animal or for surgery, and the severity and persistence of the damage is very reproducible. Second, because the animal used is the rat, the model is relatively inexpensive. Third, the inflammation induced by TNB/ethanol is long-lasting, with significant thickening of the colonic wall associated with cellular infiltration and ulcers persisting for at least 8 wk. This relatively long duration of inflammation provides a suitable period in which potential treatments can be assessed. Recent reports from this laboratory (21) and others (22) demonstrated a significant reduction of TNB-induced colonic damage

after treatment with prostaglandin analogues. A number of other groups have also carried out drug trials using the TNB model (20,23,24). This model also allows for the study of events characterizing the progression from acute to chronic inflammation. Finally, the model is histopathologically relevant, in that several of the features of human inflammatory bowel disease, particularly Crohn's disease, are present. For instance, the inflammation is transmural and includes granulomas and Langhan's-type giant cells. Ulcer morphology was strikingly similar, skip-segment ulceration and inflammation were common, and the mucosa frequently had a "cobblestone"-like appearance. Mast cell and lymphoid infiltrates and crypt distortion were also noted.

The dose of TNB and the concentration of ethanol used in most of the present study were based on the results of a dose-response study. Although the dose selected consistently produced a severe form of colonic inflammation, it did not significantly affect the long-term weight gain of the animals. Significant weight loss was only observed during the first week after administration of TNB/ethanol and with the dose used, a low rate of mortality was encountered. It should be noted that the dose of TNB/ethanol required to produce the described level of inflammation may vary with the age, strain, and sex of rat used. In studies performed in collaboration with

Table 3. Effects of Trinitrobenzenesulfonic Acid/Ethanol on Colon Wall Thickness and Granuloma Formation: Time-Course Study

Time after TNB/ethanol	Colon Wall Thickness (μm)		Animals with granulomas (%)	Sections with granulomas (%)
	Normal	Damaged		
Saline control	452 ± 17	—	0/5 (0)	0/14 (0)
Ethanol control	405 ± 16	548 ± 21*	0/19 (0)	0/60 (0)
1 wk	518 ± 21	980 ± 38*	5/16 (31)	6/36 (17)
2 wk	619 ± 36	1116 ± 46*	9/17 (53)	14/50 (28)
3 wk	452 ± 10	1091 ± 29*	13/23 (57)	16/71 (23)
4 wk	539 ± 12	926 ± 52*	2/10 (20)	2/28 (8)
5 wk	597 ± 20	1006 ± 88*	2/17 (15)	2/56 (4)
6 wk	457 ± 35	884 ± 113*	2/7 (29)	2/13 (15)
7 wk	522 ± 20	1030 ± 58*	1/6 (17)	1/13 (6)
8 wk	556 ± 81	1250 ± 122*	2/5 (40)	3/18 (17)

Colon wall thickness was measured as the distance from the mucosa to the luminal surface of the mucosa. Granulomas were observed approximately six times as often in the sections of grossly damaged regions as they were in samples of grossly normal regions. "Ethanol control" specimens were taken from rats given the 50% ethanol vehicle 1–3 wk before they were killed (grossly damaged regions were only observed up to 2 wk after ethanol administration). For the "saline control" group, rats were killed 1–8 wk after intracolonic administration of 0.9% saline. * $p < 0.01$ by Student's t-test when compared with normal samples from the same colon.

Doughton-Smith et al. (20) on male Wistar rats (200–225 g), a dose of 20 mg of TNB in 20% ethanol was found to produce inflammation and ulceration similar to that observed in the present study. Others have found a dose of 50 mg of TNB in 10% ethanol to yield a reproducible level of ulceration and inflammation (Guarner F, personal communication).

Although previous sensitization to TNB was not required for induction of colonic inflammation in this model, preliminary studies in our laboratory suggest that relapse can be induced by intracolonic administration of TNB alone (i.e., no ethanol) several weeks after challenge with TNB/ethanol. Thus, it is possible that the animals do become sensitized to the damaging effects of TNB. The ability to induce relapse by repeated intraluminal administration of TNB may provide a method to mimic another important clinical characteristic of Crohn's disease; namely, the commonly observed pattern of remission and relapse. We recently reported that the colonic inflammation induced by TNB/ethanol can be significantly reduced by prior induction of tolerance to TNB (25). This observation supports the hypothesis that there is an immunologic component to the mechanism of action of TNB in inducing chronic inflammation and ulceration.

Whether or not the administration of antigens normally found in the mammalian gut, in combination with a "barrier breaker", would yield similar results has yet to be determined. It should be noted that Sartor et al. (9) demonstrated that bacterial cell wall fragments from Streptococcus pyrogenes and Streptococci faecium were capable of eliciting chronic inflammation when injected into the bowel wall of the rat and that uptake of bacterial cell wall polymers was enhanced by acute injury to the colon (26). Studies on the carrageenan model for ulcerative colitis have also demonstrated the importance of bacterial populations in the development of experimental colitis (27).

Although the inflammation was limited to the region of the gastrointestinal tract into which the TNB was administered, similar ulceration and inflammation can also be produced in other regions. We have previously reported that oral administration of 50 mg of TNB in 0.75 ml of 50% ethanol resulted in the formation of discrete ulcers in the antral region of the stomach. These ulcers were also associated with transmural, granulomatous inflammation (28).

In conclusion, the TNB model appears to fulfill the criteria set out at the beginning of our study. It provides a simple, reproducible, and inexpensive model of chronic inflammation and ulceration of the colon. The duration of the inflammation, the composition of the inflammatory cell population, and the

ability to monitor the progression of the disease by a variety of methods suggest that this model may be of some value in the assessment of potentially therapeutic agents for the treatment of colonic inflammation. This model should also yield information relevant to the dissection of the crucial components of the pathophysiology of inflammatory bowel disease.

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